Original Article

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Development of HPTLC analysis to evaluation of Rosmarinic and Caffeic acids in *Salvia aegyptiaca* extract and analysis of its antioxidant activities

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Abstract

Phenolic acids such as rosmarinic acid (RA) and caffeic acid (CA), are the most significant phenolic compounds in Lamiaceae family, including Salvia species. The aim of this study was to evaluate the RA and CA contents of *Salvia aegyptiaca* (SA) methanol extract and its antioxidant activity. RA and CA analysis were performed by HPTLC-image analysis. UV-VIS spectrophotometry was used for the evaluation of the phenolic acid. The samples were spotted on a HPTLC plate silica gel. The toluene- ethyl acetate- formic acid was used as mobile phase. Plates were scanning at 366 nm and absorption mode was scanned. Finally, the image of plates was obtained by visualizer at 254 and 366 nm and visible wavelengths. This Method was then validated in terms of precision and accuracy at inter- day and intra-day. The extracts were screened to determine antioxidant activity using the DPPH radical scavenging assay, FRAP, ABTS, ORAC, Nitric oxide scavenging ability and cellular antioxidant activity. The RA and CA contents of extracts were 11.33±0.36 and 6.36±4.7 mg/g respectively. The SA were able to scavenge free radicals such as DPPH•, NO• and O2•–, and the ability to reduce Fe^{3+}, as well. This activity highly associated with RA and CA contents. Developed HPTLC method is rapid, simple and reliable and it is suggested to use in routine assay of *Salvia* sp. extracts containing RA and CA which are a generally marker in various plant. Moreover, SA could be considered as natural source of antioxidants.

Keywords: *Salvia aegyptiaca*, HPTLC, Rosmarinic Acid, Caffeic Acid, Antioxidant.

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1. Introduction

The genus Salvia is one of the Lamiaceae family members, which is spread all around the world (1). The 56 species have been reported in Iran (2). The Iranian common Persian name is "Maryam- Goli" and approximately 53% of them are endemics (3).

The high diversity of the *Salvia* genus and different phytochemicals lead to various pharmacological effects. They also, can be used as food preservative (4)

Some species of *Salvia* genus have industrial and pharmaceutical applications. For instance, some of them are flavoring agents and perfume additives (1, 3). In folk medicine, some *Salvia* species have been known with wide-range of therapeutic effects such as anticancer, antiviral, antimicrobial, antioxidant, and anti-inflammatory. Also, they are effective in the treatment of mental, nervous and gastrointestinal disorders (3, 5, 6). According to previous reports phenolic compounds are the most determined constitutes in *Salvia* genus. These compounds comprise caffeic

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acid (CA), rosmarinic acid and caffeic acid (7), flavones, flavonols and their glycosides derivatives (1, 5). Moreover, Abietane, labdane, ictexane, (8, 9), triterpenoids and sterols (10) are other secondary metabolite in this genus.

Salvia aegyptiaca (SA) disperses in the South of Iran, particularly Hormozgan province with local name "*Maryam-Goli Mesri*" (2). Its therapeutic effects are so considerable .It is known as digestive, carminative, antiseptic, and analgesic in Iranian folk medicine. It is also introduced as potent antibacterial and antioxidant agent (3).

In previous research, methanol and acetone extracts of SA showed antinociception and sedation effects (11). Another study indicated that SA extract was able to improve the neurohormonal disorders induced by Carbon Tetrachloride (12). Also, decoction and methanol extracts of SA were suggested as an antioxidant, anti-Alzheimer, and antidiabetic agents since they showed anticholinesterases, anti-α-amylase, and anti-α-glucosidase activities (13).

Some previous report studies have made effort to determine the major compounds of SA. For instance, methanolic extracts of SA of north and center of Tunesia were rich sources of different phenolic acids (Rosmarinic acid) and also phenolic diterpenes. Furthermore, those samples contain flavonoids (14, 15). Beside flavonoids, a coumarin and fatty acids were identified in extracts of SA collected in Qatar (16).

It seems that there is no report about phytochemical compounds of endemic SA up to now. Therefore, we were encouraged to investigate the RA and CA components in SA by using HPTLC techniques. Moreover, antioxidant activities of SA methanolic extract were analyzed by different methods.

2. Materials and methods

2.1. Chemicals

All solvents, standards, and reagents were analytical grade. HPTLC plate silica gel 60 F 254 (20×10 cm, Merck, Darmstadt, Germany); toluene; ethyl acetate (Samchun, Korea); formic acid (Biochem, France); rosmarinic acid and caffeic acid were purchased from Sigma-Aldrich, Germany. Also, DPPH, sodium nitroprusside, Griess reagent, AAPH, TPTZ DCFH-DA, ammonium persulfate, and fluorescein are belong to Sigma-Aldrich, Germany Company.

2.2. Plant material

SA was collected from Bandar Abbas (Hormozgan province) in April 2019 and identified by M. Zaefi (voucher number: MPPRC-93-2). It was deposited in medicinal plant processing research center, Shiraz University of Medical Sciences.

2.3. Sample preparation

Methanolic extract was prepared as follow; 1 gr of dry powder was dissolved in methanol, inserted in ultrasonic water bath (80 °C for 15 min), then filtered and concentrated by rotary evaporator. Subsequently, n-hexane was added four times. Afterward, methanolic phase was concentrated by using rotary evaporator and speed vacuum and finally, diluted with methanol.

RA and CA solution (0.01 mg/ml) were prepared as standard.

2.4. Chromatographic conditions

RA and CA were evaluated by HPTLC analysis, according to the method completely described in previous research (7). Briefly, Camag HPTLC (Switzerland) was used in room temperature and humidity 20 %. It was equipped with ATS4, ADC2, scanner 3, and visualizer. The ATS4 under N2 gas (5 bar pressure) was used to spotted sample on a HPTLC plate silica gel 60 F254 (20×10 cm). Plate was developed by ADC2 with following parameters: toluene- ethyl acetate- formic acid (67.72-22.90) and 9.38 % as mobile phase; plate preconditioning time 1.0 min; filling volume 10 ml; migration distance 75 mm and drying time 1.0 min.

Plates were scanned in wavelength of 366 nm and absorption mode was done by scanner 3. At the end, the image of plates was obtained by visualizer at 254, 366, and visible wavelengths. The WinCATS software recorded the results.

2.5. DPPH Free Radical Scavenging Assay

In brief, sample (12.5-3200 µg/ml) was added to DPPH (100 mM). Controls contained methanol, DPPH, and blank contains of plant ex-

tract and methanol. The mixture was incubated at 25 ºC for 30 min, and the absorbance was measured at 492 nm. The obtained data were used to determine the concentration of the extracts required to scavenge 50% of the DPPH free radicals (IC_{50}). Quercetin (3.125-1600 µg/ml) was used as a standard (17).

2.6. Nitric oxide radical scavenging

Fifty microliters of nitroprusside (10 mmol) was mixed with the samples in microplate wells. Blank contains only samples. Control contains nitroprusside and methanol. In each microplate well, Griess reagent (100 µl) was added and the microplate was incubated at 27 ºC for 150 min. After that, the absorbance was read at 542 nm (18).

2.7. FRAP (Ferric-Reducing Antioxidant Power) assay

The FRAP reagent included TPTZ solution (10 mM) in HCl (40 mM), FeCl3 (20 mM) and acetate buffer (0.3 M , pH 3.6). The fresh mixture was prepared and incubated at 37 °C. Then, 20 µl of samples and 180 µl of FRAP reagent were mixed and incubated at 37 °C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. Results reported as IC_{50} (19).

2.8. ABTS Assay

To prepare $ABTS^{\bullet+}$ radicals, ammonium persulfate solution (2.45 Mm) was added to ABTS solution (7 Mm) and kept in dark for 16 h. This $ABTS^{\bullet+}$ solution was diluted with ethanol to gain an absorbance of 0.7± 0.02 at 734 nm. $ABTS^{\bullet+}$ solution and samples were mixed and the absorbance was recorded at 734 nm. The antioxidant activity was calculated by the following formulas: E= (1-(At/Ac)) ×100, which At and Ac are the absorbance of tested samples and ABTS•+, respectively (19).

2.9. Oxygen radical absorbance capacity assay (ORAC)

ORAC assay was done by Polar star omega device (BMG LABTECH GmbH, Germany). The assay mixture contained AAPH (240 Mm), fluorescein (10 Nm), and 25 µl of the sample or phosphate buffer as the blank. The quercetin was used as standard. The fluorescence of this mixture was recorded every 90 sec per cycle using fluorescent filters. Different concentrations of Trolox were used to give a standard curve to compare the ORAC values of various samples. The data were analyzed using data analysis software, MARS, linked with Omega reader control software. The difference between the "area under the fluorescence decay curve" (AUC) of blank and each sample were expressed as Trolox Equivalents (TE) (20).

2.10. Cellular antioxidant activity (CAA)

HepG2 cells (6×10^4 cells / well) were incubated in microplate for 24 hr. After 24 h seeding, the growth medium was removed and cells were washed gently 3 times with PBS. Triplicate wells were treated with DCFH-DA solution (50 µl) and with either quercetin standards or samples.

The microplate was incubated at 37 °C for 60 min. Then the liquid was removed and cells were washed 3 times by PBS. After that, the last phase was washed and the liquid was removed and discarded. Finally, AAPH was added to all wells and the plate was read on the Polar star omega (21).

3. Results and Discussion

According to previous investigations *S. aegyptica* exhibited different pharmacological effects such as CNS depressant (11), antimicrobial (22), and antioxidant (1).

Salvia specious are known as natural antioxidants since they are enriched in polyphenols compounds. These compounds are responsible for different pharmacological effects as well as nutritional value (14). Nepetoideae is subfamily of Lamiaceae which is rich in RA, a well-known phenolic acid. A broad range of biological activities have been related to RA, comprise astringent, antioxidant, anti-inflammatory, anti-mutagenic, antibacterial, and antiviral. High amount of RA and its various biological effects make it as a proper marker to standardize natural product belong to sage family (23).

In this study, HPTLC method was developed to quantity of RA and CA in methanolic extracts of SA.

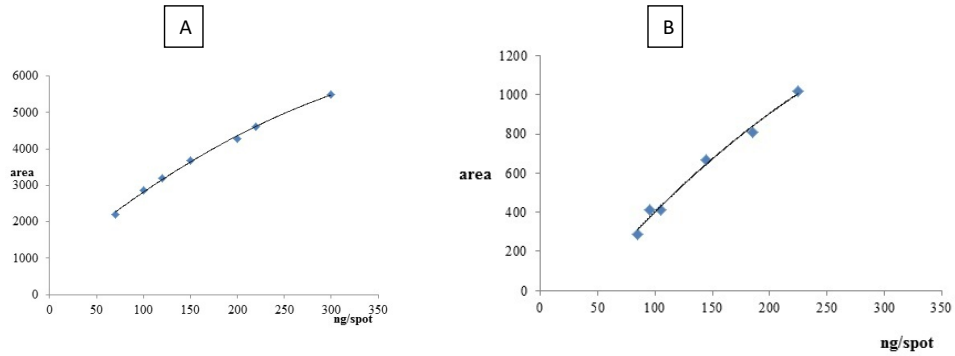

Figure 1. Calibration curve of rosmarinic acid (A) and Caffeic acid (B).

Calibration curves of RA at concentrations of 70, 100, 120, 150, 200, 220 and 300 ng/spot and 85, 95, 105, 145, 185 and 225 ng/spot for CA were obtained in amount per fraction mode. TLC scanner 3 was used for evaluation of RA and CA at 366 nm and all of measurements were repeated 3 times daily on 3 different days. The values of R_f in the development solvent system was 0.12±0.01 for RA and 0.27±0.01 for CA. Polynomial regression (Figure 1. A and B) analysis lead to a calibration curve with the equation of y=-0.021 x^2+21.885 x+837.73 and r2=0.9976 for RA and y=-0.0078 x^2+7.3889 x-259.38 for CA with r2=0.9906. Inter-day and intra-day variability of data was presented in the Table 1 & 2. Amounts of RA and CA based on mg/g for dried plant were mentioned in the Table 3.

Different methods such as solid state differential pulse voltammetry, UHPLC, ESI/MSn and HPLC were used to analysis of RA content in herbal extracts. Nevertheless, HPTLC methods were used in few report to quantify the RA in Lamiaceae plant (23). For instance, Fecka et al., reported polyphenol contents along with RA in some Lamiaceae spices, i.e., thyme, marjoram, lemon balm, and mint samples (23). In another study, polyphenolic content including RA of *Satureja hortensis*, were analyzed by HPTLC but this method was not validated (24).

Analysis of data at inter-day and intra-day showed that the mentioned method has a proper accuracy for RA and CA determination. The ranges of 4.52-45.72 mg/g and 3.19-464.64 mg/g were obtained for RA and CA, respectively. In a previous study, Altan et al. developed HPTLC method for the quantification of RA in seven salvia speciouses. The results showed that RA content (w/w %) in 1.5 mg/ml of samples. These contents were 1.2% in *S. officinalis*, 7.3% in *S. heldreichiana*, 7.2% in *S. tomentosa*, 3.8% in *S. triloba*, 4.1% in *S. sclarea*, 4.2% in *S. dichroantha,* and 5.0% in *S. candidissima*. Also, reported RA content in other *Salvia* specious were *S. compressa* (45.72±1.6 mg/g), *S. mirzayanii* (8.50±0.46), *S. santolinfolia* (7.34±0.15), *S. macilenta* (5.64±0.18 mg/g), and *S. sharifii* (4.52±0.11 mg/g) (7).

Analysis of phytochemical composition of *S. officinalis*, *S. elegans*, and *S. greggii* decoctions by UHPLC-DAD-ESI-MS showed that CA and derivates were 39.8±0.9, 74.1±0.5 and 20.8±0.3 mg/g of extract, respectively (25).

In a previous study, SA from two origins, at three different phenological stages were analysed by HPLC. In this report, methanolic extracts of SA contain phenolic acids, phenolic diterpenes and flavonoids. These phenolic acids comprise gallic acid, p-hydroxy benzoic acid, caffeic acid, ferulic acid and RA. RA was found as major phe-

Table 1. Precision and accuracy results at inter- day and intra-day for rosmarinic acid.

Amount (ng)	Intra-day		Inter-day	
	Accuracy %	Precision %	Accuracy %	Precision %
50	6.59	4.32	3.96	2.31
170	1.04	4.06	-0.48	1.34
250	0.01	4.55	1.54	1.44

Table 2. Precision and accuracy results at inter- day and intra-day for Caffeic acid.

Amount (ng)	Intra-day		Inter-day	
	Accuracy %	Precision %	Accuracy %	Precision %
125	13.5	1.6	-1.2	2.62
165	4.60	1.94	-1.10	4.39
205	12.8	5.35	-3.83	6.54

nolic compounds. They proposed that the amount of RA was depend on the time of harvesting (14).

Furthermore, RP-HPLC analysis of MeOH extracts of *S. verticilllata* ssp. verticillata and *S. verticillata* ssp. amasiaca showed that they possess high levels of RA (37.1 and 24.83 mg/g, respectively) and also, (CA 9.82 and 8.69 mg/g, respectively) (26).

Moreover, quantitication of CA and RA in Lamiaceae species with TLC-densitometric method was investigated by Janicsák and coworkers (27). Their analysis revealed the presence of RA in all species of the subfamily Nepetoideae; however, in the subfamily Lamioideae RA was not detected. On the other hand, with the exclusion of three Lamioideae species, CA was identified in all examined taxa. Values of RA and CA were obtained as 0.01 to 9.30 mg/g and 0 to 0.62 mg/g, respectively.

Table 3. Amounts of Rosmarinic acid and Caffeic acid based on mg/g for dried plant.

Rosmarinic acid		Caffeic acid	
(mg/g ± SD, n=3)	CV %	(mg/g ± SD, n=3)	CV %
11.33±0.36	3.2	6.36±4.7	3.7

Different methods have been used to analysis of RA in various extracts. These methods comprise UHPLC, ESI/MSn, and HPLC (28-30). Though, there are limited number of report reveal the efficacy of HPTLC methods for the determination of RA in Lamiaceae herbs (23).

It seems that HPTLC helps to analysis samples in short time since it is possible to analysis several samples on a single plate. Also, fingerprint for certain compounds can be optimized so easily. This method would be applied in industrial scale for routine analysis of many natural products(23).

Antioxidants are able to prevent or slow cellular damage caused by free radicals and oxidative stress. Phenolic compounds are known as antioxidant and play critical role in oxidative stress-related diseases (31). In DPPH assay, the ability of extract to transfer an electron or hydrogen atom to DPPH• radical was described (32). Based on our results, it seems that this extract is able to scavenge DPPH• radical which is related to phenolic content.

In DPPH assay, DPPH radical react with hydrogen donor antioxidant; consequently, purple color of DPPH convert to yellow. Antioxidant activity was related to intensity of yellow color. The IC_{50} of methanolic extract was 72.68±0.77 µg/ml. The IC_{50} of quercetin is 26.51±0.06 µg/ml (Table 4). In other method, FRAP assay, interaction of TPTZ-Fe (III) complex and sample resulted in TPTZ-Fe (II) complex formation. So in this assay, reducing power of extract was evaluated. Phenolic compounds are responsible of reducing power activity of this extract. The IC_{50} of extract was 84.10±0.84. In nitric oxide radical scavenging assay, nitric oxide radicals (NO•) are able to interacts with oxygen and produce nitrite ions (NO-). The result of nitric oxide scavenging ability of 200

Table 4. Antioxidant activity of *Salvia aegyptiaca* extract.

Sample	DPPH (IC_{50}, µg/ ml)	ABTS (IC_{50}, µg/ ml)	FRAP (IC_{50}, µg/ ml)	Nitric oxide scavenging ability % (200 µg/ml)
Salvia Aegyptiaca	72.68±0.77	78.71±0.056	84.10±0.84	41.8±0.018
Q Uercetin	26.51±0.06	26.35±0.028	18.53±0.12	89.96±0.007

µg/ml concentration of extract was 41.8±0.018 % (Table 4).

According to results of DPPH, FRAP, and ABTS assays, methanolic extract was active. SA has both hydrophobic and hydrophilic compounds. It seems that hydrophilic compounds, especially water-soluble phenolic acids in methanolic extract, are responsible of its antioxidant activities.

According to the results of ORAC assay, TE were 0.4±0.03 and 1.05±0.04 for methanolic extract of SA and quercetin, respectively. These results show the ability of extract to scavenge the produces alkoxyl and alkyl peroxyl radicals. Since in this assay, AAPH is a generator of these radicals (33).

The results of CAA assay (EC50) were 18.19 and 1.09 for methanolic extract and quercetin, respectively. In this assay, a fluorescent probe is used to monitor the inhibition of peroxyl radical inside the cell (34). The results of CAA analysis suggested this extract as a potent antioxidant in cells. So, this extract would be valuable source in future studies. In the biochemistry of Lamiaceae family, CA play critical role and occurs mostly present as dimer form which known as RA (35).

RA has been known as an astringent, antioxidant, anti-inflammatory, anti-mutagenic, antibacterial, and antiviral agent (2). As RA is potent antioxidant, so it can be used to alleviate oxidative stress-related disorders (3-5).

In previous studies, different Salvia genus has been reported as powerful antioxidant such as *Salvia elegans* Vahl., *Salvia greggii* A. Gray, and *Salvia officinalis* L. (25) *Salvia pilifera* (36), *Salvia eriophora* (37) *Salvia kronenburgii* Rech. f., *Salvia euphratica* (38), and *Salvia rosmarinus* F (38). It seems that similarly to the other Salvia species, SA is a respected source of natural antioxidant and could be used as pharmaceutical ingredients targeting oxidative-related diseases.

5. Conclusion

According to our results, HPTLC method was suggested to the quantity of RA and CA in herbal extracts especially this technique was successful in *Salvia* sp. methanol extracts analysis. This method was simple, precise, specific and sensitive. Our results also offered aerial parts of SA as proper source of natural antioxidant.

Conflict of Interest

None declared.

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